

The present invention relates to the nucleotide sequence of the gene encoding the horse cytokine GM-CSF, to expression vectors containing it, and to its use as adjuvant in equine vaccination and as nonspecific immunity stimulant.

The documents cited here are incorporated by reference into the present application. (All documents cited herein, and all documents cited in documents cited herein are hereby incorporated herein by reference)

The first discovery of a granulocyte-macrophage colony-stimulating factor (GM-CSF) dates from 1977 (Burgess A.W. et al. J. Biol. Chem. 1977, **252**, 1998-2003). It is the murine GM-CSF, purified from mouse lung culture supernatants.

The biological activities of GM-CSF have been demonstrated by the work carried out on the murine and human GM-CSFs (Clark S.C. et al. Science 1987, **230**, 1229; Grant S.M. et al. Drugs 1992, **53**, 516).

GM-CSF has many physiological roles (Dy M. in "Les cytokines" Cavaillon J.-M., 1996, ed. Masson, Paris, France, 43-56). In particular, GM-CSF stimulates the production, the development and the formation of colonies of granulocytes, macrophages, eosinophils and megakaryocytes. GM-CSF induces in particular a macrophagic cytotoxicity, stimulates antibody-dependent cytotoxic activity (ADCC) and the recruitment of leukocytes at the level of the sites of inflammation.

The GM-CSFs from various animal species have already been identified.

The sizes of the nucleotide sequences encoding the known GM-CSFs from various species vary from 381 to 432 nucleotides. The human and murine nucleotide sequences have a degree of homology of 69%. The degree of homology is 54% at the level of the amino acid sequence (Cantrell

M.A. et al. Proc. Natl. Acad. Sci. USA 1985, **82**, 6250-6254). However, this homology does not allow any cross-activity between the two human and murine species (Metcalf D. et al. Blood 1986, **67**, 37-45).

5       The administration of heterologous GM-CSF, that is to say obtained from a species other than the one treated, does not make it possible to obtain an optimum adjuvant effect, in particular a stimulation of the activity of the haematopoietic cells and a substantial  
10      increase in the immune response.

Up until now, it has not been possible to identify the equine GM-CSF. Yet this cytokine is of great interest for therapeutic and vaccinal applications for use in horses.

15       The applicant has succeeded in isolating and sequencing the equine GM-CSF gene. This gene was isolated after polymerase chain reaction (PCR) with the aid of the oligonucleotides described in the examples.

20       The equine GM-CSF gene has a size of 432 nucleotides (SEQ ID No. 8 and Figure 1) and encodes a protein of 144 amino acids (SEQ ID No. 9 and Figure 1). The protein encoded by this gene exhibits a homology of at least 75% with the GM-CSF polypeptide sequences of other animal species.

25       The subject of the present invention is therefore an isolated DNA fragment encoding equine GM-CSF, e.g. a fragment comprising SEQ ID No. 8. Its subject is also the DNA fragment having or consisting essentially of this sequence.

30       The subject of the present invention is also an isolated DNA fragment encoding the amino acid sequence SEQ ID No. 9.

The invention covers the equivalent nucleotide sequences of equine or synthetic origin, that is to say

the nucleotide sequences encoding a protein of equivalent functionality and specificity in horses. The nucleotide sequences which differ by the degeneracy of the genetic code will of course be included. In particular, DNA  
5 sequences having an homology equal or greater than 90%, particularly than 92%, preferably than 95% with SEQ ID No. 8, are equivalent sequences.

An object is also DNA fragments comprising such a nucleotide sequence encoding the equine GM-CSF, e.g.  
10 according to SEQ ID No. 8 or a sequence encoding the amino acid sequence SEQ ID No. 9, this nucleotide sequence being associated, in the form of a fusion, with the nucleotide sequence encoding at least one immunogen or at least one immunogenically active fragment or at  
15 least one epitope of an immunogen. The DNA fragment then does not comprise a stop codon between the sequence encoding GM-CSF and the associated immunogen encoding sequence. For instance, referring to SEQ ID No. 8, the coding sequence inserted ends at nucleotide 432, and does  
20 not include the stop codon.

The subject of the present invention is also the isolated equine GM-CSF protein or polypeptide, e.g. that encoded by the nucleotide sequence SEQ ID No. 8 or by the  
25 equivalent of the latter as defined above.

The subject of the present invention is also the equine GM-CSF protein having the amino acid sequence SEQ ID No. 9.

The equine GM-CSF protein has a size of 144 amino  
30 acids. However, the present invention also comprises the proteins, protein fragments and polypeptides of equine origin or which are synthetic, having a size greater or equal than or less than these 144 amino acids, as well as the recombinant proteins (having one or more

substitutions, deletions or additions) and the fusion proteins, as long as their biological activity (for the part which is common to GM-CSF) is substantially equivalent to that of the natural equine GM-CSF protein  
5 in vivo in horses and their species-specificity is not modified. Are encompassed as equivalents any of the amino acid sequences encoded by any of the equivalent nucleotide sequences as defined above.

The subject of the present invention is also a pure  
10 preparation of equine GM-CSF protein.

The subject of the present invention is also the expression vectors containing, as insert, any of the above defined DNA fragments or nucleotide sequences, in particular the equine GM-CSF gene (SEQ ID No. 8) or an  
15 equivalent thereof as defined above, as well as any of the nucleotide sequences encoding any of the above defined amino acid sequences. Also, the vector may further comprise a nucleotide sequence encoding at least one immunogen or at least one immunogenically active  
20 fragment or at least one epitope of an immunogen, which can be or not associated under the form of a fusion as described above.

The nucleotide sequence may be inserted into conventional *in vitro* expression systems of viral origin,  
25 such as Baculovirus, in particular propagated on insect cells, or cells of prokaryotic origin (for example *Escherichia coli*) or eukaryotic origin, in particular yeasts, especially *Saccharomyces cerevisiae*, mammalian eukaryotic cells, especially hamster cells (for example  
30 hamster ovary cells or CHO) and horse cells. The invention therefore also covers expression systems transformed by a sequence according to the invention, the equine GM-CSF proteins thus produced and their use as adjuvant for vaccine and nonspecific immunity stimulant.

Preferably, the sequence according to the invention is introduced into *in vivo* expression vectors under conditions allowing the expression, in horses, of a functional equine GM-CSF protein, and possibly a nucleotide sequence encoding at least one immunogen or at least one immunogenically active fragment or at least one epitope of an immunogen. These expression vectors may be plasmids, viral vectors, such as poxviruses, for example the vaccinia virus, avipoxviruses (canarypox, fowlpox), including the species-specific poxviruses (swinepox, raccoonpox and camelpox), adenoviruses and herpesviruses, such as the equine herpesviruses.

The term plasmid is intended to cover any DNA transcription unit in the form of a polynucleotide sequence comprising the sequence of the equine GM-CSF gene and the elements necessary for its expression *in vivo*. The circular plasmid form, supercoiled or otherwise, is preferred. The linear form also falls within the scope of this invention.

Each plasmid comprises a promoter capable of ensuring, in the host cells, the expression of the gene inserted under its control. It is in general a strong eukaryotic promoter and in particular a cytomegalovirus early promoter CMV-IE, of human or murine origin, or optionally of other origin such as rat or guinea pig. More generally, the promoter is either of viral origin or of cellular origin. As a viral promoter other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous Sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the promoter specific to the gene. As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter, or alternatively the actin promoter.

When several genes are present in the same plasmid, they may be provided in the same transcription unit or in two different units.

5 The plasmids may also comprise other transcription  
regulating elements such as, for example, stabilizing  
sequences of the intron type, preferably intron II of the  
rabbit  $\beta$ -globin gene (van Ooyen et al. Science, 1979,  
206: 337-344), signal sequence of the protein encoded by  
the tissue plasminogen activator gene (tPA; Montgomery et  
10 al. Cell. Mol. Biol. 1997, 43: 285-292), and the  
polyadenylation signal (polyA), in particular of the  
bovine growth hormone (bGH) gene (US-A-5,122,458) or of  
the rabbit  $\beta$ -globin gene.

The invention also covers the immunogenic  
15 compositions and the vaccines comprising the equine  
GM-CSF protein according to the invention, and at least  
one immunogenic or vaccinal preparation of equine  
pathogen, and a veterinarily acceptable excipient or  
vehicle. The notion of immunogenic preparation covers  
20 here any preparation capable, once administered to  
horses, of inducing an immune response directed against  
the equine pathogen considered, a response which is  
increased by the presence of the GM-CSF protein. It is  
preferably a vaccinal preparation capable of inducing an  
25 effective protection or a degree of protection against  
this pathogen, a degree of protection which is increased  
here by the presence of the equine GM-CSF protein. The  
immunogenic and vaccinal preparations intended in the  
invention cover all the known types, such as inactivated,  
30 attenuated live, subunit and recombinant (using an *in*  
*vivo* expression vector, in particular of viral or plasmid  
origin). As was seen above, the GM-CSF protein may be  
added as such to the immunogenic or vaccinal preparation  
to form, in the presence of a veterinarily acceptable

excipient or vehicle, an immunogenic composition or a vaccine ready to be administered. It is also possible to envisage combining the GM-CSF protein with a prolonged-release system designed to gradually release the protein.

5       According to a more advantageous mode of the invention, it is however preferable to express the GM-CSF protein *in vivo* using an *in vivo* expression vector as described above. In this case, it is also preferable that the immunogenic or vaccinal preparation is also of the  
10 recombinant type, based on the use of an *in vivo* expression vector, of the same type or of a different type. It is also possible to envisage using the same *in vivo* expression vector, comprising and expressing at least one equine pathogen immunogen and the equine GM-CSF  
15 protein.

The advantages of the use of GM-CSF during vaccinations are in particular the reduction in the dose of immunogen or of vector or DNA used. Furthermore, in some animals which do not respond when administered with  
20 a customary vaccine, the use of GM-CSF allows the stimulation of the immune response and its increase up to a protective level.

The present invention therefore preferably covers the immunogenic compositions and the vaccines comprising:  
25 • an *in vivo* expression vector containing a nucleotide sequence encoding an equine GM-CSF under conditions allowing the expression, in horses, of a functional equine GM-CSF protein,  
• at least one *in vivo* expression vector containing at  
30 least one nucleotide sequence encoding at least one equine immunogen, it being understood that this vector or some or all of these vectors (when there are several vectors encoding various immunogens) may also constitute the GM-CSF vector (say the vector comprises

at least the GM-CSF sequence and an immunogene sequence), and

- a veterinarily acceptable vehicle or excipient.

According to a preferred mode of the invention, the  
5 invention covers the immunogenic compositions and the  
vaccines of the DNA type, comprising a plasmid encoding  
and expressing the equine GM-CSF according to the  
invention and at least one other plasmid encoding and  
expressing an equine immunogen or an immunologically  
10 active fragment derived therefrom. Examples of plasmid  
constructs which contain an equine immunogen and which  
can be used in the invention are given in patent  
application WO-A-9803198. The invention also covers the  
DNA vaccines comprising a plasmid encoding and expressing  
15 simultaneously the equine GM-CSF and at least one equine  
immunogen.

The invention covers all the equine pathogens. There  
may be mentioned more particularly equine herpesvirus  
type 1 or type 4 (and preferably the invention provides  
20 for combination of both types), equine influenza virus,  
tetanus, *Borrelia burgdorferi*, Eastern, Western and  
Venezuelan equine encephalites, rabies virus. For the  
subunit vaccines and the recombinant vaccines, the equine  
immunogens are preferably selected from the group  
25 comprising the glycoproteins gB, gC and gD of the equine  
herpesvirus type 1 or type 4, the haemagglutinin (HA) and  
the nucleoprotein (NP) of the equine influenza virus, the  
C subunit fragment of the tetanus toxin, the *Borrelia*  
*burgdorferi* OspA protein, the Eastern, Western and  
30 Venezuelan equine encephalites E2 and C genes, the rabies  
virus G gene.

The subject of the present invention is also  
nonspecific immunity stimulating compositions, that is to  
say which can be used as a general immunity stimulant in

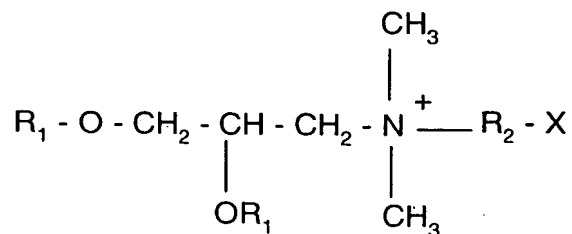


horses. These compositions are administered in the presence or in the absence of a declared pathology, in general independently of any vaccine, in order to reinforce the immune defenses in horses. These  
5 compositions comprise GM-CSF according to the invention, in all the forms described above, protein or recombinant, preferably recombinant (in vivo viral or plasmid expression vector), and a veterinarily acceptable excipient or vehicle. The characteristics of these  
10 vectors have already been described.

The nonspecific stimulating compositions and the immunogenic compositions and the vaccines according to the invention may also comprise one or more immunity adjuvants, in particular selected from those normally  
15 used in equine vaccination against the pathogens (valencies) considered. The stimulating compositions and the immunogenic compositions and conventional vaccines (inactivated, attenuated live, subunit) may thus comprise, as conventional adjuvant, compounds of the  
20 carbomer or aluminum hydroxide type, or may be formulated in the form of an oil-in-water emulsion. For the stimulating compositions and the immunogenic compositions and recombinant vaccines based on a viral expression vector, there may be mentioned the oil-in-water  
25 emulsions.

According to a preferred mode of the invention, for the stimulating compositions of the plasmid type and the immunogenic compositions and vaccines of the plasmid type, the plasmid encoding and expressing the equine  
30 GM-CSF, the plasmid encoding and expressing GM-CSF and at least one equine immunogen, as well as the mixtures of plasmids containing the latter and at least one plasmid encoding an equine immunogen, may be advantageously formulated in a novel fashion with a cationic lipid

containing a quaternary ammonium salt, of formula:



in which  $\text{R}_1$  is a saturated or unsaturated linear  
5 aliphatic radical having 12 to 18 carbon atoms,  $\text{R}_2$  is  
another aliphatic radical containing 2 or 3 carbon atoms,  
and X a hydroxyl or amine group

It is preferably the DMRIE (N-(2-hydroxyethyl)-N,N-  
dimethyl-2,3-bis(tetradecyloxy)-1-propanammonium; WO-A-  
10 9634109), preferably coupled with a neutral lipid, DOPE  
(dioleoylphosphatidylethanolamine), to form preferably  
DMRIE-DOPE. Preferably, the recombinant vector mixture  
with this adjuvant is made immediately before use and  
preferably, before its administration to the animal, the  
15 mixture thus produced is allowed to form a complex, for  
example over a period ranging from 10 to 60 minutes, in  
particular of the order of 30 minutes.

When DOPE is present, the DMRIE:DOPE molar ratio  
preferably ranges from 95:5 to 5:95, more particularly  
20 1:1.

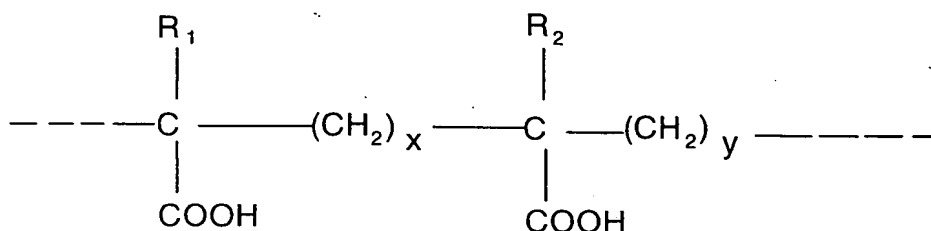
The plasmid:DMRIE or DMRIE-DOPE adjuvant weight  
ratio may range in particular from 50:1 to 1:10, in  
particular from 10:1 to 1:5, preferably from 1:1 to 1:2.

According to another advantageous mode of the  
25 invention, for the stimulating compositions of the  
recombinant type and the immunogenic compositions and  
vaccines of the recombinant type (viral vector or  
plasmid), it is possible to use, as adjuvant, polymers of  
acrylic or methacrylic acid or copolymers of maleic  
30 anhydride and of alkenyl derivative. The polymers of

acrylic or methacrylic acid crosslinked in particular with polyalkenyl ethers of sugars or of polyalcohols are preferred. These compounds are known by the term carbomer (Pharmeuropa vol. 8, No. 2, June 1996). Persons skilled  
5 in the art can also refer to US-A-2,909,462 (incorporated by reference) describing such acrylic polymers crosslinked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being replaced  
10 with unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such  
15 as methyl. The products sold under the name Carbopol<sup>®</sup> (BF Goodrich, Ohio, USA) are particularly appropriate. They are crosslinked with an allyl saccharose or with allylpentaerythritol. Among them, there may be mentioned Carbopol<sup>®</sup> 974P, 934P and 971P.

20 Among the copolymers of maleic anhydride and of an alkenyl derivative, the EMAs<sup>®</sup> (Monsanto) are preferred which are copolymers of maleic anhydride and ethylene, linear or crosslinked, for example crosslinked with divinyl ether. Reference may be made to J. Fields et al.,  
25 Nature, **186**: 778-780, 4 June 1960 (incorporated by reference). From the point of view of their structure, the polymers of acrylic or methacrylic acid and the EMAs<sup>®</sup> preferably consist of basic units of the following formula:

30



in which:

- $\text{R}_1$  and  $\text{R}_2$ , which are identical or different, represent  
5 H or  $\text{CH}_3$
- $x = 0$  or  $1$ , preferably  $x = 1$
- $y = 1$  or  $2$ , with  $x + y = 2$

For the EMAs<sup>®</sup>,  $x = 0$  and  $y = 2$ . For the carbomers,  
10  $x = y = 1$ .

The dissolution of these polymers in water leads to  
an acidic solution which will be neutralized, preferably  
to physiological pH, to give the adjuvant solution into  
which the actual vaccine will be incorporated. The  
carboxyl groups of the polymer are then partly in  $\text{COO}^-$   
15 form.

Preferably, a solution of carbomer or of EMA<sup>®</sup> is  
prepared in distilled water, preferably in the presence  
of sodium chloride, the solution obtained being at acidic  
pH. This stock solution is diluted by adding it to the  
20 required quantity (in order to obtain the desired final  
concentration), or a substantial part thereof, of water  
loaded with NaCl, preferably physiological saline (NaCl  
9 g/l), in one or more portions with concomitant or  
subsequent neutralization (pH 7.3 to 7.4), preferably  
25 with NaOH. This solution at physiological pH will be used  
as it is to mix with the immunogenic or vaccinal  
preparation, in particular stored in lyophilized, liquid  
or frozen form.

The polymer concentration in the final vaccine  
30 composition will be 0.01% to 2% W/V, more particularly

0.06 to 1% W/V, preferably 0.1 to 0.6% W/V.

Another object of the invention is a method of immune stimulation and/or immunisation and/or vaccination of equine species, wherein a stimulating, immunogenic or vaccine composition according to the invention is administered to an animal from equine species, in particular a horse. Administration is preferably done via parenteral route, such as intramuscular, intradermal or subcutaneous route. One or more administrations can be done. In particular, in case of vaccination, administration is done each time the vaccine is administered.

The quantity of DNA used in the stimulating compositions and the immunogenic compositions and vaccines according to the present invention is between about 10 µg and about 2000 µg, and preferably between about 50 µg and about 1000 µg, for a given plasmid. Persons skilled in the art will have the competence necessary to precisely define the effective dose of DNA to be used for each therapeutic or vaccination protocol.

If a live vector is used, doses may be between  $10^4$  and  $10^{10}$  Pfu (plaque forming unit) preferably between  $10^6$  and  $10^8$  pfu.

For a composition containing GM-CSF protein, doses may be from 1 µg to 5 mg, preferably from 50 µg to 1 mg.

The dose volumes may be in particular between 0.5 and 5 ml, preferably between 2 and 3 ml.

The invention will now be described in greater detail with the aid of embodiments taken as nonlimiting examples and referring to the drawing in which:

**Figure 1:** Sequences of the equine GM-CSF gene and protein

**Figure 2:** Restriction map of the plasmid pJP097

**Sequence listing SEQ ID for the constructs of the present**

**invention**

SEQ ID No. 1 Oligonucleotide JP705

SEQ ID No. 2 Oligonucleotide JP706

SEQ ID No. 3 Oligonucleotide JP729

5 SEQ ID No. 4 Oligonucleotide JP730

SEQ ID No. 5 Oligonucleotide JP731

SEQ ID No. 6 Oligonucleotide JP734

SEQ ID No. 7 Oligonucleotide JP735

10 SEQ ID No. 8 Sequence of the equine GM-CSF gene (see Figure 1)

SEQ ID No. 9 Sequence of the horse GM-CSF protein (see Figure 1).

**EXAMPLES**

15 All the constructions of the plasmids were carried out using the standard molecular biology techniques (cloning, digestion with restriction enzymes, synthesis of a single-stranded complementary DNA, polymerase chain reaction, extension of an oligonucleotide with a DNA  
20 polymerase and the like) described by Sambrook J. et al. (Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York. 1989). All the restriction fragments used for the present invention, as well as the various polymerase chain  
25 reaction (PCR) fragments were isolated and purified using the "Geneclean<sup>®</sup>" kit (BIO101 Inc. La Jolla, CA).

**Example 1: Preparation of the total RNA of horse lymphocytes stimulated in vitro by mitogens**

30 Horse blood was collected on a tube containing EDTA by taking blood from the jugular vein. The mononucleated cells were arrested by centrifugation on a Ficoll gradient and then cultured in a Petri dish 60 mm in diameter. The horse mononucleated cells in culture were

then stimulated either with concanavalin A (conA) (final concentration of about 5 µg/ml) or with phytohaemagglutinin (PHA) (final concentration of about 10 µg/ml). After stimulation, the "ConA" and "PHA" lymphoblasts were harvested by scraping the culture dishes, and the total RNA of these cells was extracted using the "mRNA isolation kit for white blood cells" (Boehringer Mannheim/Roche Cat # 1 934 325).

10 **Example 2: Isolation of the gene encoding the equine GM-CSF**

The oligonucleotides JP075 and JP076 were synthesized and have the following sequences:

JP705 (SEQ ID No. 1) (20 mer)

15 5'TGGGCACTGTGGYCTGCAGC3'

JP706 (SEQ ID No. 2) (17 mer)

5'AGCATGTGRATGCCATC3'

These oligonucleotides were used with the 5'/3'RACE kit (Boehringer Mannheim/Roche Cat # 1 734 792) in order to generate the 3'RACE clones 6S4, 6W6 and 6W7. The 3' consensus sequence established from these 3 clones was used to synthesize the oligonucleotides JP729, JP730 and JP731 which will serve for the generation of the corresponding 5'RACE clones:

25 JP729 (SEQ ID No. 3) (21 mer)

5'AGCTCCCAGGGCTAGCTCCTA3'

JP730 (SEQ ID No. 4) (21 mer)

5'CCCTGTTTGTACAGCTTCAGG3'

JP731 (SEQ ID No. 5) (21 mer)

30 5'TGTTGTTTCAGAAGGCTCAGGG3'

The corresponding 5'RACE clones obtained were the clones 7D2 and 7D10. The consensus sequences generated from the 3'RACE clones and the 5'RACE clones were used to amplify the entire sequence of the equine GM-CSF gene

according to the reverse transcriptase technique followed by a PCR. The total RNA extracted from the horse lymphocytes stimulated by ConA or by PHA (Example 1) served as template for the complementary DNA first strand synthesis. This complementary DNA first strand was produced by extension of the oligonucleotide p(dT)15 (Boehringer Mannheim/Roche Cat # 814 270). The single stranded complementary DNA obtained was then used as template for a PCR reaction with the following oligonucleotides:

JP734 (SEQ ID No. 6) (44 mer)

5'CATCATCATGTCGACGCCACCATGTGGCTGCAGAACCTGCTTCT3'

and JP735 (SEQ ID No. 7) (41 mer)

5'CATCATCATGCGGCCGCTACTTCTGGGCTGCTGGCTTCCAG3'

in order to amplify a PCR fragment of about 500 base pairs (bp). This fragment was purified by agarose gel electrophoresis (= fragment A).

**Example 3: Construction of the plasmid pJP097 and sequence of the equine GM-CSF gene**

Fragment A (Example 2) was digested with NotI and SalI and the NotI-SalI fragment thus obtained was ligated into the plasmid pVR1012 (Hartikka J. et al. Human Gene Therapy, 1996, 7, 1205-1217), previously digested with NotI and SalI, to give the plasmid pJP097 (5334 bp, Figure 2). The NotI-SalI fragment cloned into this plasmid was completely sequenced. This sequence (SEQ ID No. 8), which encodes a protein of 144 amino acids (SEQ ID No. 9) is the horse GM-CSF cytokine (= equine GM-CSF) represented in Figure 1.

**Example 4: Biological activity in vitro of the product of the equine GM-CSF gene**

CHO-K1 cells (hamster ovary cells, accessible from



the strain depository American Type Culture Collection under the access number CCL-61) were cultured in minimum essential medium or MEM (Gibco-BRL) in Petri dishes 60 mm in diameter and transfected with 5 µg of plasmid pJP097, previously complexed with 10 µl of LipofectAmine PLUS<sup>®</sup> (Cat# 10964-013, Gibco-BRL, Cleveland, OH, USA). The conditions for forming the DNA/LipofectAmine<sup>®</sup> complexes and for transfecting the cells were those recommended by the supplier (Gibco-BRL). 48 hours after the transfection, the culture supernatants are harvested and frozen.

Bone marrow cells collected from pigs are cultured in a semisolid Methocult medium (Cat# H4230 from StemCell Technologies). These cultures are then supplemented or otherwise (negative control) with 10 µl of the supernatant of the cells transfected with the plasmid pJP097. Two independent transfections were carried out with the plasmid pJP097, encoded pJP097 T1 and pJP097 T2. Each supernatant (10 µl diluted 1/10) is tested in parallel in 3 culture dishes. The negative control consists of a CHO culture supernatant. After 14 days of culture, the dishes are examined for the formation of colonies of macrophages, and the colonies which may be present are counted.

The supernatants of CHO cells transfected with the plasmid pJP097 gave the following results:

Plasmid/dilution supernatant	No. of dishes	Mean number of colonies per dish	Standard deviation
Control	3	0	0
pJP097 T1 (eGM-CSF)	3	12	2
pJP097 T2 (eGM-CSF)	3	15	0

These results show that the product of the equine GM-CSF gene expressed by the plasmid pJP097 has a GM-CSF-type activity on cells in vitro.

5

**Example 5: Preparation of the plasmids according to the invention**

For the preparation of the plasmids intended for the vaccination of horses, it is possible to use any technique allowing a suspension of purified plasmids to be obtained. These techniques are well known to persons skilled in the art. The production of the plasmids is carried out by culturing *Escherichia coli* K12 bacteria transformed with the plasmids according to the invention.

15 There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on cesium chloride gradient in the presence of ethidium bromide as described in Sambrook J. et al. (Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY. 1989).

20 Reference may also be made to patent applications WO-A-95/21250 and WO-A-96/02658 which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the purposes of vaccine

25 production, the plasmids are resuspended so as to obtain solutions at high concentration ( $> 2$  mg/ml) which are compatible with storage. To do this, the plasmids are resuspended either in ultrapure water, or in TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

30

**Example 6: Manufacture of the vaccines according to the invention and administration**

The stock of plasmid pJP097 is diluted in TE buffer, in physiological saline or in PBS buffer, and mixed with

various vaccinal plasmids expressing protective immunogens. These plasmids may be, for example, those cited in the examples of patent application PCT WO 98/03198.

5       The horses are vaccinated with doses of 100 µg, 250 µg or 500 µg per plasmid.

      The various mixtures of "immunogenic" plasmids and of the plasmid pJP097 "equine GM-CSF" thus obtained are coadministered by the intramuscular route (syringe +  
10   needle) into the neck or breast muscles. In this case, the vaccinal doses are injected in a volume of 2 ml.

      The intramuscular injections may also be carried out using a liquid jet injection apparatus (without needle) which drives a dose of e.g. 0.5 ml. If necessary, several  
15   successive administrations may be made in the same animal in order to inject volumes greater than 0.5 ml. The successive shots are then made apart, so that the areas for injection are separated by about 1 to 2 centimeters.

      The injections may also be carried out by the  
20   intradermal route using a liquid jet injection apparatus (without needle) delivering a dose of 0.2 ml at 5 sites (0.04 ml per site of injection) (for example "PIGJET"<sup>®</sup> Endoscopic apparatus, Laon, France).

      The horses are typically vaccinated using two  
25   injections of mixtures of plasmids according to the invention carried out with a 4-5 week's interval.

**Example 7: Formulation of the plasmids according to the invention**

30       The mixture of "immunogenic" plasmids and of the plasmid pJP097 is diluted in TE buffer, in physiological saline and in PBS buffer so as to obtain a concentration of 1 mg/ml. A solution of DMRIE-DOPE at 0.75 mM is prepared by taking up a lyophilisate of DMRIE-DOPE in a

suitable volume of sterile H<sub>2</sub>O.

The formation of plasmid DNA-lipid complexes is achieved by diluting, in equal parts, the 0.75 mM DMRIE-DOPE solution with the DNA solution at 1 mg/ml. The DNA solution is gradually introduced, with the aid of a  
5 seamed 26G needle, along the wall of the vial containing the cationic lipid solution so as to avoid the formation of foam. Gentle shaking is carried out as soon as the two solutions have been mixed. A composition comprising  
10 0.375 mM DMRIE-DOPE and 500 µg/ml of DNA is finally obtained.

It is desirable for all the solutions used to be at room temperature for all the operations described above. The DNA/DMRIE-DOPE complex formation is allowed to take  
15 place at room temperature for 30 minutes before immunizing the animals as described in Example 6.

It should be clearly understood that the invention defined by the appended claims is not limited to the  
20 specific embodiments indicated in the description above, but encompasses the variants which depart from neither the scope nor the spirit of the present invention.